Urinary Metabolites of 3a,4,5,6,7,7a-Hexahydro-3-(1-methyl-5-nitro-1H-imidazol-2-yl)-1,2-benzisoxazole in the Dog

W. J. A. VANDENHEUVEL [*x], B. H. ARISON [*], T. W. MILLER [*], P. KULSA [*], P. ESKOLA [*], H. MROZIK [*], A. K. MILLER [‡], H. SKEGGS [‡§], S. B. ZIMMERMAN [‡], and B. M. MILLER [‡]

Received December 21, 1978, from *Merck Sharp & Dohme Research Laboratories and ‡Merck Institute for Therapeutic Research, Rahway, NJ 07065. Accepted for publication March 27, 1979. §Merck Sharp & Dohme Research Laboratories, West Point, PA 19486.

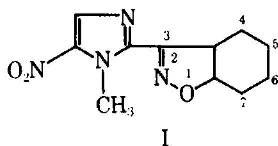
Abstract □ The antiprotozoal drug 3a,4,5,6,7,7a-hexahydro-3-(1-methyl-5-nitro-1H-imidazol-2-yl)-1,2-benzisoxazole (I), which exhibits activity against trypanosomiasis, is also antibacterial *in vivo*. Since the urine from a dog dosed with I showed a broader spectrum of antibacterial activity than I itself, metabolites from this urine were isolated and partially characterized. The metabolites were mono- and dihydroxy-substituted species with the hydroxyl groups on carbons 4–7 of the hexahydrobenzisoxazole ring. These observations led to the synthesis of several such hydroxy derivatives of I, and their properties fully supported the proposed positions of metabolic hydroxylation. One synthetic compound, the 6,7-*cis*-dihydroxy compound, exhibited higher antibacterial activity against *Salmonella schottmuelleri* in mice and greater trypanocidal activity *in vivo* against *Trypanosoma cruzi* (Brazil strain) than I.

Keyphrases □ 3a,4,5,6,7,7a - Hexahydro-3 - (1-methyl-5-nitro-1H-imidazol-2-yl)-1,2-benzisoxazole—urinary metabolites, antibacterial activity, antiprotozoal activity, dog □ Antiprotozoal agents—3a,4,5,6,7,7a-hexahydro-3-(1-methyl-5-nitro-1H-imidazol-2-yl)-1,2-benzisoxazole, urinary metabolites, antibacterial activity, antiprotozoal activity, dog □ Benzisoxazoles—3a,4,5,6,7,7a-hexahydro-3-(1-methyl-5-nitro-1H-imidazol-2-yl)-1,2-benzisoxazole, urinary metabolites, antibacterial activity, antiprotozoal activity, dog

3a,4,5,6,7,7a-Hexahydro-3-(1-methyl-5-nitro-1H-imidazol-2-yl)-1,2-benzisoxazole (MK-436, I) exhibits antiprotozoal activity (1), including activity against *Trypanosoma cruzi*[1]. The evaluation of I for mutagenicity was hampered by the fact that the compound was antibacterial in the host-mediated mouse test at levels of 200 mg/kg or higher but not at 10 mg/ml in the Ames *et al.* (2) spot test utilizing *Salmonella typhimurium* G46[2]. These results aroused the suspicion that antibacterial metabolites were produced by the mouse, especially since incubation of I with liver homogenate fraction S-9, prepared by reported procedures (3), produced antibacterial activity not observed with I alone. Since mouse and dog urine from animals dosed with I showed a broader antibacterial spectrum (and higher potency) than I, the present study was undertaken to characterize the canine urinary metabolites of I to allow their synthesis for biological testing.

EXPERIMENTAL

Dog urine was collected for metabolite isolation. The dog was dosed with I at 1000 mg/kg po in two divided doses per day for 2 days. This regimen was repeated 2 weeks later; the two 48-hr postdose urine collections were pooled to yield 2800 ml. The pooled urine was extracted twice with equal volumes of methylene chloride. The extracts were combined, concentrated to dryness under reduced pressure, dissolved in methanol, and centrifuged to remove insoluble components.



I

The methanol was removed *in vacuo*, and 150 mg of the residue was dissolved in benzene and chromatographed on a column containing 100 g of silica gel[3] packed in benzene. The column was developed with a stepwise gradient of methanol in benzene as follows: 400 ml of benzene, 800 ml of benzene containing 5% methanol (v/v), and then 600 ml of benzene containing 10% methanol. The column was developed at 5 ml/min, and 20-ml fractions were collected and assayed against a highly sensitive bacterium, *Bacillus subtilis* MB964.

The most active fractions occurred toward the end of the 5% methanol development, and they were pooled and concentrated to dryness to yield 68 mg of residue. The residue was dissolved in 1.7 ml of ethanol, and 100-μl portions were subjected to preparative TLC on silica gel plates[4]. The plates were developed with benzene–methanol (9:1) and then examined under shortwave UV light and by bioautography against *B. subtilis*. Ten distinct bands were observed under UV light, and five were active against *B. subtilis*. Four major bands [R_f 0.27 (active), 0.32 (inactive), 0.37 (active), and 0.42 (inactive)] were scraped off the plates and extracted with benzene–methanol (5:1). These extracts (designated Metabolite Fractions A–D) were examined by direct probe mass spectrometry, combined GLC–mass spectrometry, and NMR spectroscopy.

n-Butylboronate derivatives were prepared by allowing aliquots of samples dissolved in acetone to react with excess *n*-butylboronic acid[5] for 1 hr at room temperature. Trimethylsilylation was carried out by treating aliquots of samples with bis(trimethylsilyl)acetamide[6] or bis-(trimethylsilyl)acetamide-d_{18}[7] (to form the perdeutero derivatives) and pyridine (2:1) for 1 hr at 60°.

Mass spectra were obtained[8] using a 70-ev ionizing potential, a 3.5-kv accelerating potential, a 60-μamp trap current, and a 250° source temperature. GLC conditions were: 1.8-m × 3-mm i.d. spiral glass column at 205°; 3% OV-101 on 80–100-mesh acid-washed and silanized diatomaceous earth[9]; flash heater and molecular separator temperatures, 250°; and helium flow rate, 30 ml/min. NMR spectra were obtained[10] with samples dissolved in deuterochloroform.

The spent urine from the extraction was concentrated to 1150 ml under reduced pressure, adjusted to pH 4.5 with 1 *M* HCl, mixed with 30 ml of an enzyme preparation containing sulfatase and glucuronidase[11], and then incubated at 37° for 18 hr. This mixture was extracted with 2 liters and then with 1 liter of methylene chloride. The extracts were combined, concentrated to dryness under reduced pressure, dissolved in 15 ml of methanol, and centrifuged to remove insoluble components. Aliquots of this solution (Metabolite Fraction E) were examined also by mass spectrometric-based methods.

In vivo evaluations of I and 6,7-*cis*-dihydroxy-I (Compound G) were conducted by suspending the test compounds evenly in a 1% methylcellulose aqueous vehicle to the desired concentrations. Albino male mice[12]

[1] C. M. Malanga, J. Conroy, and A. C. Cuckler, Merck Sharp & Dohme Research Laboratories, Rahway, NJ 07065, personal communication.
[2] H. Skeggs, unpublished data.

[3] Silica gel G, Brinkmann.
[4] Quanta/Gram QIF plates, Quantum Industries.
[5] Applied Science Laboratories.
[6] Supelco.
[7] Merck Sharp & Dohme of Canada.
[8] LKB model 9000.
[9] Gas Chrom P, Applied Science Laboratories.
[10] Varian HA-100D equipped with a Fourier transform accessory.
[11] Glusulase, Endo Laboratories.
[12] Carworth Farms.

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Table I—Mass Spectrometric Behavior of I and Related Compounds

I		Dihydroxy Metabolite C			Dihydroxy Metabolite C n-BuB[c]		Monohydroxy Metabolite			6,7-*cis*-Dihydroxy Synthetic G		
Ion, *m/e*	Intensity[a]	Ion, *m/e*	Δ[b]	Intensity	Ion, *m/e*	Intensity	Ion, *m/e*	Δ	Intensity	Ion, *m/e*	Δ	Intensity
M, 250	100	M, 282	32	35	M, 348 (^{11}B)	100	M, 266	16	73	M, 282	32	61
					347 (^{10}B)	25						
M − 17, 233	42	M − 17, 265	32	30	M − 17, 331 (^{11}B)	19	M − 17, 249	16	61	M − 17, 265	32	43
		M − 35, 242	—	20						M − 35, 247	—	70
		M − 43, 239	—	6	M − 57, 291 (^{11}B)	73	M − 43, 223	—	25	M − 43, 239	—	22
M − 45, 205	17	M − 45, 237	32	5			M − 45, 221	16	11	M − 45, 237	32	8
							M − 61, 205	0	10			
M − 55, 195	36	M − 87, 195	0	90	M − 153, 195	57	M − 71, 195	0	83	M − 87, 195	0	89
169	21	169	0	8	169	13	169	0	21	169	0	16
153	70	153	0	100	153	64	153	0	100	153	0	100
107	18	107	0	22	107	11	107	0	30	107	0	31

[a] Relative to base peak (100). [b] Change in *m/e* relative to I. [c] *n*-Butylboronate derivative.

(CF-1 strain), 18–22 g, were inoculated subcutaneously with ~2 × 10^4 *T. cruzi* B[13]; the inoculum was parasitemic mouse blood diluted with Hoppe-Chapman solution (0.75% NaCl and sodium citrate plus 0.2% glucose).

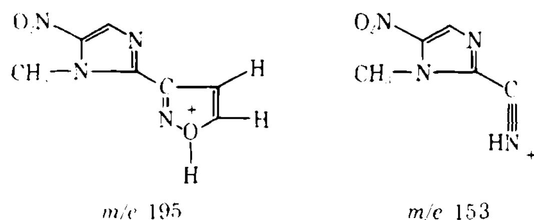
Twenty-four hours after inoculation and for 3 successive days thereafter, each mouse was dosed by gavage with 0.2 ml of the test suspension at the desired concentration; there were two groups of five mice each per treatment. Infected, sham-treated (five groups), and sham-infected, sham-treated (two groups) controls were included. Mortality and percent with parasitemia were noted through Day 50 postinoculation. All infected controls became parasitemic, and 83% died.

For the mouse protection study, Charles River CDI female mice, 21 g, were injected intraperitoneally with 0.5 ml of a broth dilution of a 15-hr culture of *Salmonella schottmuelleri* MSD 3010. The challenge dose contained about 1 × 10^7 colony-forming units, estimated to be 11 times the number of organisms that should kill 50% of the infected, nontreated mice (LD_{50}). The test compounds were suspended evenly in 5% dimethylformamide and were administered by gavage immediately after injection and again 6 hr later. There were five mice at each of the fourfold drug concentrations tested. On the 7th day after infection, the test was considered complete; the survival records of that day were used to calculate the ED_{50} values, the amount of compound that should protect 50% of the infected, treated mice.

RESULTS AND DISCUSSION

Mass spectrometric data for I are presented in Table I. The molecular ion is the base peak with other intense, pertinent ions at *m/e* 233 (M − 17; possibly loss of hydroxyl from the adjacent methyl and nitro groups), 195 [M − 55, loss of C_4H_7 (C-4–C-7 with all but one of the associated hydrogen atoms)], and 153.

The canine metabolite fractions were subjected to direct probe mass spectrometry. Without exception, each appeared to contain a drug-related compound possessing a molecular weight of 282, suggesting introduction of two oxygen atoms into I. Mass spectrometric data for Metabolite Fraction C are presented in Table I. Based on a comparison with the fragmentation pattern of I, the metabolic transformations occurred on C-4–C-7 of the hexahydrobenzisoxazole or "cyclohexane" ring and did not involve either heterocyclic ring. Although the spectra from the four fractions all showed the same molecular ion (*m/e* 282), their relative intensity patterns varied significantly, suggesting that the four zones



[13] Culture was isolated from a Brazilian patient in 1942 by T. S. Hauschka and maintained by mouse passage at the National Institutes of Health from which R. Hewitt (Lederle Laboratories) obtained subsequent passage material in March 1948. A passage culture was provided to the authors in 1969 and cryogenically preserved until use.

contain compounds in which two hydroxyl groups are substituted isomerically on positions C-4–C-7 of the cyclohexane ring.

Each metabolite fraction also was subjected to derivatization using *n*-butylboronic acid (4, 5) followed by direct probe mass spectrometry (Table I). These products all exhibited *m/e* 348 as a molecular ion, with fragment ions of *m/e* 331 (M − 17), 291, 195, 169, 153, and 107; the relative intensities of the ions varied from isolate to isolate. The increases of 66 mass units in molecular weight and the $^{10}B/^{11}B$ ratio (1:4) at *m/e* 347/348 demonstrate formation of monocyclic boronate esters and require the presence of 1,2- or 1,3-(*cis*)-diol systems in the metabolites. The ion of *m/e* 291 (M − 57) arises *via* loss of the butyl radical (C_4H_9); prominent ions that do not contain boron (*m/e* 195, 169, 153, and 107) are also found in the spectra of I and underivatized metabolite fractions.

These data demonstrate that the compounds in the TLC zones are metabolites of I: isomeric, dihydroxy-substituted species in which the hydroxyl groups are present on positions 4–7[14]. GLC–mass spectrometry of the trimethyl- (and perdeuterotrimethyl-) silylated metabolite fractions not only supported the dihydroxylation proposal [molecular ions of 426/444 (d)[15]] but showed that each fraction is multicomponent in nature (Fig. 1 and Table II).

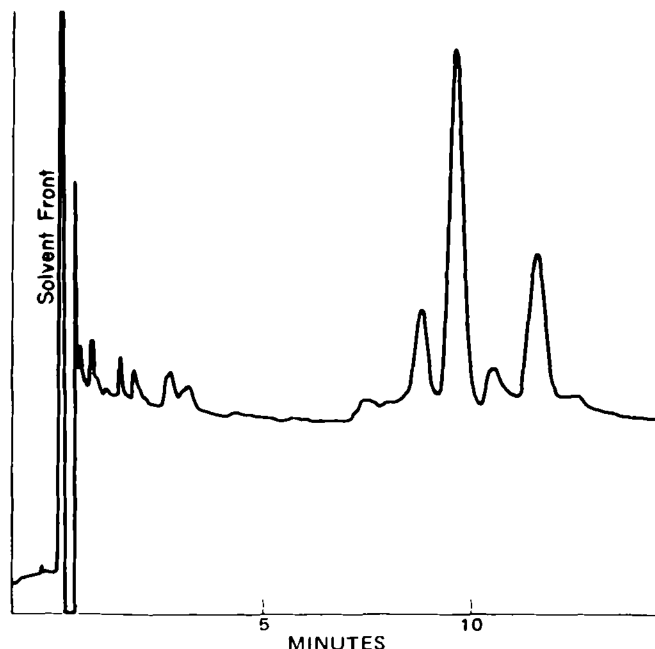


Figure 1—*Gas chromatogram of trimethylsilylated Metabolite Fraction C. Each of the four major peaks results from a metabolite of I. Column conditions are given in* Experimental *section.*

[14] Studies carried out on a methylene chloride extract of the urine of mice treated with I demonstrated that cyclohexane ring hydroxylation also occurred in this species.
[15] Value for trimethylsilyl-d_9 derivative.

Table II—GLC Retention Behavior of Metabolites of I and of Related Compounds

Sample	Retention Time[a]	Extent of Hydroxylation
A	0.92	Dihydroxy
	1.21	Dihydroxy
B	0.76	Dihydroxy
	0.90	Dihydroxy
	1.20	Dihydroxy
C	0.91	Dihydroxy
	1.00	Dihydroxy
	1.09	Dihydroxy
	1.21	Dihydroxy
D	0.97	Dihydroxy
E	0.70	Monohydroxy
	0.76	Dihydroxy
	0.81	Monohydroxy
	0.91	Dihydroxy
	0.97	Dihydroxy
	1.20	Dihydroxy
F[b]	1.19	Dihydroxy
G[c]	1.19	Dihydroxy

[a] Retention times (trimethylsilyl derivatives) relative to the major component of C. Column conditions are given in *Experimental* section. [b] 5,6-*cis*-Dihydroxy. [c] 6,7-*cis*-Dihydroxy.

NMR spectroscopy provided general support for the mass spectrometric conclusion that the metabolites were dihydroxylated on the cyclohexane ring. Although the isolates are admittedly multicomponent in nature and impure, the imidazole ring could be excluded as a possible hydroxylation site since the chemical shifts of the *N*-methyl and nuclear proton were essentially unchanged from those in the parent drug. Compared with the NMR spectrum of I, the metabolites showed new signals in the region associated with HC attached to oxygen. Only partial characterizations were attempted since the isolates were mixtures of two or more closely related isomeric structures.

Fraction E, containing metabolites originally present as conjugates, was examined by direct probe and GLC-mass spectrometry, free and also following trimethylsilylation (Table II). These studies demonstrated that it contained a mixture of mono- and dihydroxy (two and four, respectively) metabolites with the hydroxyl group(s) present on the cyclohexane ring. Mass spectrometric data for one of the two monohydroxy metabolites are presented in Table I. The molecular ion is m/e 266, 16 mass units greater than that of I. Many fragment ions in the spectrum possess the same m/e values (*i.e.*, 195, 169, 153, and 107) as those found in the spectra of I and its dihydroxy metabolites, indicating the hydroxylation occurred at C-4–C-7. As required, the molecular ions for the trimethylsilyl derivatives of the monohydroxy metabolites were found at m/e 338/347 (d). Mono- and dihydroxylation of the cyclohexane ring are reminiscent of the work by Elliott *et al.* (6), who reported that, in the rabbit, the urinary cyclohexane metabolites are the glucuronides of cyclohexanol and *trans*-cyclohexane-1,2-diol. Renwick and Williams (7) also demonstrated that these alcohols (free and conjugated) are cyclohexylamine metabolites in several species. The metabolic hydroxylation of cyclohexyl groups was discussed by Testa and Jenner (8).

Because of the large number of possible dihydroxy I isomers and the complicated nature of the metabolite fractions, complete structural elucidation of the metabolites was not undertaken. Based on available information, however, several dihydroxy I derivatives (Table II) were synthesized[16]. Their mass spectrometric and other physical properties demonstrated that the metabolic transformation of I does, indeed, involve hydroxylation of the hexahydrobenzisoxazole ring (Table I, comparison of the mass spectra of Metabolite Fraction C and Compound G[17]). Efficacious doses (no deaths; no parasitemia) of I and G against an experimental *T. cruzi* B infection in mice were 62.5 and 15.6 mg/kg po × 4 days, respectively. The ED_{50} values of I and G against *S. schottmuelleri* infections in mice were 3.0 and 1.25 mg/mouse (150 and 62.5 mg/kg × 2 doses), respectively. The approach chosen in this study—structural characterization of biologically active metabolites followed by synthesis of bioactive compounds based on this information—has been validated.

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[16] Unpublished data.

[17] Although the GLC and mass spectrometric data for several metabolites and the synthesized dihydroxy compounds are similar, it is not possible now to state unequivocally whether there are cases of identity.